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Title: Pneumococcal vaccines.

The invention relates to the field of vaccines against microbial infections and especially bacterial vaccines, in particular to pneumococcal vaccines.

*Streptococcus pneumoniae* (pneumococcus, *S. pneumoniae*) is an important pathogen, which causes significant morbidity and mortality throughout the world. *S. pneumoniae* is a major cause of invasive diseases such as meningitis, bacteremia, and pneumonia, as well as non-invasive diseases like acute otitis media and sinusitis (1). In young children, the pneumococcus is often part of the normal nasopharyngeal flora. Especially during the first two years of life, children are colonised with novel strains of pneumococci. Children colonised with *S. pneumoniae* develop more often acute otitis media than children who are not colonised (2, 3, 4).

The precise molecular mechanisms through which the pneumococcus invades and damages host tissues are not fully understood. For many years, the polysaccharide capsule has been recognised in the art as the major virulence factor and, consequently, an important vaccine candidate (for review, see 5, 6). The current pneumococcal vaccine strategies focus on the use of conjugates, in which a limited number of different capsular polysaccharides are linked to a carrier protein (7,8). Although the results of early trials look promising, problems still arise since large-scale vaccination over time generally leads to a shift in serotype distribution towards capsular types that are poorly immunogenic or not included in the vaccine. Such a shift may be enhanced by the frequent horizontal exchange of capsular genes, as described by several investigators (9, 10, 11).

Over the last few years, much attention has been focused on the role of pneumococcal proteins in pathogenesis and protection. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered to be interesting components for future conjugate vaccines. Such proteins are able to switch the immune response against the polysaccharides present in the vaccine from T-cell independent to T-cell dependent, through which the antibody response towards the polysaccharides may be increased and a memory response will be provided. In addition, such proteins should provide protection against colonisation and infection

with *S. pneumoniae* strains whose capsular polysaccharides are not included in the vaccine.

The protective abilities of various (virulence) proteins have been investigated previously. Immunisation of pneumolysin (12), pneumococcal surface protein A (PspA) (13, 14,15) pneumococcal surface adhesin A (PsaA) (16), and neuraminidase (17) clearly confer protection in animals.

In the literature various polynucleotides of *S. pneumoniae* and polypeptides predicted to be encoded by said nucleotides have been reported and the use of these compounds in vaccines and medicinal preparation has been contemplated, for instance in WO 97/37026 and WO 98/18930. These publications however, do not identify any functional protein let alone a vaccine based on a functional protein. These publications are further silent in respect of proteins that when used in vaccines are able to elicit an immuneresponse let alone that they are able to elicit any protective, more in particular opsonophagocytic activity.

The publications further do not disclose any information regarding cross reactivity towards various strains of *S. pneumoniae* in a relevant vertebrate host. Furthermore these publications do not describe the protease maturation protein of *S. pneumoniae*. Another publication that relates to the present invention is WO 00/06737. This publication discloses a pool of several hundreds of proteins. Most of these proteins, including the protein described in the present invention have not been tested for their immuneresponsive properties, opsonophagocytic activity or cross reactivity.

The present invention identifies surface-associated proteins from *S. pneumoniae* with immune-protective properties, more in particular opsonophagocytic activity. Furthermore the present invention provides the use of these proteins as vaccine components and their use in conjugate vaccination strategies. The invention further provides for antibodies which express opsonophagocytic activity and methods for their production, as for example detailed in the experimental part.

It has now been found that a surface-associated protein of *S. pneumoniae* can be used in the preparation of a vaccine against micro-organisms and especially *S. pneumoniae*. This surface protein is present in a large number of strains of *S. pneumoniae*.

The invention acc rdingly relates to a vaccine or medical preparation comprising a protease maturation protein of S. pneumoniae and/or a fragment thereof and/or a homologous and/or functionally homologous protein and/or fragment thereof for the treatment of microbial infections and especially of *S. pneumoniae* infections and for the generation of antibodies in an immunised or vaccinated in a vertebrate host and which expresses opsonophagocytic activity against *S. pneumoniae* and infections thereof. The invention also relates to the use of protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the treatment of a *S. pneumoniae* infection and/or colonisation and to the use of a protease maturation protein of *S. pneumoniae* or a fragment thereof or a recombinant or synthetic protein or fragment or functionally homologous protein or fragment thereof as a carrier for inducing prophylactic protection against other micro-organisms including viruses.

In this description and the appending claims treatment encompasses and generally is the prophylaxis of infections.

Surface-associated proteins were isolated or purified from the *S. pneumoniae* strains FT231 and EF3296, respectively, using either the SB14 extraction procedure or the Triton X114 extraction procedure as further illustrated in the working examples herein-below. The proteins and polypeptides were purified in relatively high concentrations, as shown by two-dimensional SDS-PAGE. Extracts from either strain resulted in a highly homologous protein profile as demonstrated by computer-assisted analysis. Since both extraction procedures resulted in comparable protein profiles, the SB14 extraction procedure was used for further experiments.

Hyperimmune serum antibodies were raised against the pneumococcal surface-associated proteins of *S. pneumoniae* strains FT231 and EF3296, respectively. To confirm the presence of surface-exposed proteins in the fraction, the sera were tested for the recognition of components at the surface of pneumococcal whole cells. Immuno-cytometric experiments demonstrated the recognition of components exposed at the surface of the homologous pneumococcal strains by the hyperimmune sera. Heterologous immuno-cytometric analysis demonstrated that the serum-recognition of components at the surface of the two strains display partial overlap as the level of fluorescence of the bacteria using the homologous serum was greater than th fluorescence level using the heterologous serum. In addition,

components at the surface of eleven other pneumococcal strains, which display ten distinct genotypes and represent eight clinically important serotypes, were invariably recognised by the hyperimmune sera. The strains which have tested are described in more epidemiological detail by Hermans et al. (10).

Hyperimmune rabbit sera raised against the surface-associated pneumococcal proteins in the phagocytosis assay as described by Alonso Develasco et al. (5) have been analysed. The *in-vitro* opsonophagocytic activity of the serum is presumed to correlate with *in-vivo* protection against *S. pneumoniae*. The opsonophagocytic activity of the hyperimmune sera was high using the homologous pneumococcal strains. The specificity of the serum opsonophagocytic activity was determined using seven genotypically distinct pneumococcal strains, representing seven serotypes that cause most infections in young children and two strains of the genetically closely related species *S. bovis* and *Enterococcus faecalis*, respectively. The hyperimmune rabbit sera were invariably opsonically active against the pneumococcal strains. In contrast, the serum opsonophagocytic activity was very low using *S. bovis* and *E. faecalis*. This means that *S. bovis* and *E. faecalis* are not recognised by the serum. Apparently these organisms have insufficient homology to *S. pneumoniae* for serological recognition.

All immunodominant proteins were cut from two-dimensional acrylamide gels. Protein characterisation was performed using mass spectrometric analysis (Maldi-tof) to analyse trypsine fragments on the amino acid level. In addition, monospecific hyperimmune rabbit serum antibodies were raised against the acrylamide-embedded proteins. The monospecific hyper-immune sera were used to identify the cellular localisation of the proteins by immuno-electron microscopy and to determine the capacity of these proteins to elicit opsono-phagocytosis.

Blast and/or Blastp computer programs were used for comparison of the sequence of the protein isolated from *S. pneumoniae* with known sequences in various databases. In this program the Expect value (E-value) is a parameter that describes the number of hits that can be expected just by chance when searching a database. The E value is a measurement for the random background noise that exists from a match between two sequences. To decide whether or nbot a protein is functionally homologous with Pmp, a homology cut-off value is defined as an E-value of $10^{-10}$. A

protein with an E-value of more than $10^{-10}$ is not considered sufficient homologous to Pmp from *S. pneumoniae*.

Sub B1 One of the proteins revealed to be homologous to a polypeptide encoded by nucleotide sequence 7632-8597 on contig 33 of *S. pneumoniae* (Figure 1). This ORF was identical to ORF 414 of *S. pneumoniae* in the WIT-system. Details about the WIT system can be found on http://wit.mcs.anl.gov/ and on the website of The Institute for Genomic Research, Rockville USA updated on April 7, 1999.

Sub B2 Since this pneumococcal polypeptide was related to protease maturation protein *Lactobacillus paracasei* (Swiss Prot acc. nr. Q02473) (Figure 2), and *Lactococcus lactis subspec. lactis* (Swiss Prot acc. nr. P15294) (Figure 3) and *Lactococcus lactis subsp. cremoris* (Swiss Prot acc. nr. P14308) (Figure 4) it was designated the protease maturation protein (Pmp) of *S. pneumoniae*. Also the molecular weight of the protein cut from the acrylamide gel corresponds with the molecular weight of Pmp.

This protein has various interesting characteristics with respect to its use in conjugate vaccines.

The immuno-electron microscopy using the monospecific rabbit antibodies raised against Pmp demonstrated that this protein was surface-associated.

The opsonophagocytic activity of the monospecific anti-Pmp rabbit antibodies was measured using the homologous pneumococcal strain, as well as seven genotypically distinct pneumococcal strains, representing seven serotypes that causes most infections in young children and two strains of the genetically closely related species *S. bovis* and *E. faecalis*, respectively. The anti-Pmp rabbit antibodies were invariably opsonically active against the pneumococcal strains. In contrast, the serum opsonophagocytic activity was very low using *S. bovis* and *E. faecalis*. These data show that Pmp has the ability to elicit immune protection, which is a major requisite with respect to its use as a vaccine component. Thus, not only the existence of the protein has been demonstrated, also its potential function and properties have been adequately established, which distinguishes the present invention over the art.

DNA sequence analysis of the *Pmp* genes of the homologous pneumococcal strain, as well as fifteen genotypically distinct pneumococcal strains, representing fourteen serotypes that cause most infections in young children demonstrated very limited variation. This is an important feature of Pmp with respect to its use as a

vaccine component, and of the present invention in general, as it will guarantee immunological cross reactivity.

Phenotypic variation is an important mechanism allowing bacterial pathogens to adapt to different host environments. In *S. pneumoniae*, phenotypic variation due to alterations in cell-surface structures can be detected as spontaneous, reversible changes in colony morphology. Such alterations result in opaque and transparent colonies within single strains. The relationship of several previously identified cell-surface structures to phenotypic variation has recently been described (18). The transparent phenotype incorporates significantly more surface-exposed phosphorylcholine. In addition, the expression of three choline-binding proteins (Cbp) also varies in the phenotypic variants. The expression of autolysin LytA, is lower in opaque variants as compared to transparent variants, pneumococcal surface protein PspA is present in higher amounts in opaque variants, and CbpA is present in higher amounts in transparent variants. Such phenotypic changes also result in alterations in virulence phenotype. The opaque phenotype has decreased ability to colonise the nasopharynx as compared to the transparent phenotype (19). In addition, the survival time of mice after intraperitoneal challenge of the opaque phenotype is decreased as compared to the transparent phenotype (20)

Pmp is predominantly present in transparent colony variants of *S. pneumoniae*. Since these variants are prone to colonise the nasopharynx in animal models (21), immunisation with conjugate vaccines containing Pmp or Pmp components will enhance the removal of colonising pneumococci from the nasopharynx.

The determination of the function of Pmp in *S. pneumoniae* has been based on the homology of the protein with Pmp proteins of other bacterial species. The function of the Pmp proteins of other bacterial species is generally the activation of certain proteases. The most important keys to the use of Pmp in vaccines is the surface exposure of Pmp, whereby Pmp is available to the immunesystem and the elicitation of opsonophagocytic activity as shown in the opsonophagocytosis assay.

Pmp has been identified herein as a conserved protein. This means that Pmp is expressed in many, if not all strains of *S. pneumoniae*. Pmp has been shown to have surface xposure and to elicit ps nophagocytic activity. These characteristics of Pmp enable the use of this protein and protein fragments or functional equivalents

thereof in the preparation of the vaccine in such manner that the vaccine can be used against nearly all strains of *S. pneumoniae*. As Pmp is depicted as the protease maturation protein *of S. pneumoniae* and as this protein has a function as a protease activator, it is therefor easily envisaged that the protease activator proteins of other bacterial species, especially of the genus *Streptococcus*, will fulfil a major role in pathogenesis. Similar protease activators from other species, for instance from *Meisseria*, can likewise be used in vaccine preparations. These homologues and functional homologues of Pmp can thus be used in the preparation of a vaccine for other bacterial species than *S. pneumoniae*. The present invention therefor also encompasses the homologues and functional homologue equivalent proteins of Pmp and fragments and their use in vaccine preparations.

In a preferred embodiment of the invention the protein or fragments thereof used in the preparation of the vaccine, is the Pmp or a (functional) homologous fragment thereof of *S. pneumoniae* strain FT231 or strain EF 3296.

It is likewise possible to employ a fragment of Pmp for the preparation of a vaccine. A fragment is a polypeptide with an amino acid sequence which is functionally similar to the corresponding section of the protein. In principle any fragment of Pmp can be used. A preferred fragment is an oligopeptide that contains one of the characterising parts or active domains of the protein. The fragment of Pmp can be (part of) an anchoring fragment, an antigenic fragment or a fragment that is (part of) a receptor binding site or an antibody binding site or combinations thereof. The Pmp or the fragment or the functional equivalent thereof can be obtained by recombinant techniques or by chemical synthesis of Pmp oligopeptides. Synthetic oligopeptides based on or derived from Pmp can for instance be obtained by conventional pepscan technology. The use of Pmp or a (homologous) fragment or a (homologous) functional equivalent thereof as a carrier in other vaccines is also encompassed by the invention. When Pmp expresses certain strongly immunogenic properties, these properties can be used by employing Pmp as a carrier. Pmp then serves to induce an immune response to a bad immunogen such as a protein or a sugar of other bacterial or viral pathogens. This strategy is useful in conjugate vaccine strategies. In an embodim nt the protease maturation protein or (homologous) fragment or (homologous) functional equivalent thereof is used as a carrier protein, preferably in a conjugate vaccine strategy.

In a preferred embodiment of th invention, the fragment is an anchoring fragment, an antigenic fragment or a functional equivalent fragment thereof or a functional equivalent for a receptor binding site or an antibody binding site.

A fragment of Pmp in general will consists of an oligopeptide of at least 5 amino acids, preferably at least about 8, but oligopeptides with 10-15 amino acids are preferred. These fragments can also be used in the from of tandem oligopeptides or dimerised oligopeptides.

The protein or (functional) fragment that is used in the preparation of the vaccine can be a partially purified protein, a purified protein or fragment of Pmp.

In order to obtain a vaccine that can be administered, the protein is brought into a form that is suitable for this purpose. To this end, the protein can be conjugated with a carrier protein. Carrier proteins that can be used in this invention are in general conventional carriers and as such are well known in the art. The vaccine can likewise also comprise adjuvants and other additional components to further ensure the proper functioning of the vaccine. These additional components are generally known by the skilled man.

In a preferred embodiment of the invention, the composition comprising the protein or the fragment is therefore combined with an adjuvant and/or a carrier. From this composition a vaccine is prepared which is used in the preventive vaccination against *S. pneumoniae*. A more preferred embodiment of the invention comprises protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the preventive treatment of a *S. pneumoniae* infection.

The invention further provides for a method for the preparation of a vaccine against S. pneumoniae. The method comprises the steps of preparing or isolating the protein or the fragment or homologue or functional homologue of the protein or fragment, determining the immunogenic response by raising antibodies against the protein or the fragment or homologue or functional homologue of the protein or fragment and testing the antibodies for activity. The method according to the invention also encompasses the recombinant or synthetic production of the protein or the fragment or homologue or functional homologue of the protein or fragment and the subsequent steps to the preparation of the vaccine.

In general, in this invention, when a protein or a fragment thereof is described, the protein and the fragment encompass the Pmp of *S. pneumoniae* or a

fragment thereof, a homologous protein or fragment or a homologous or functional homologous protein or fragment thereof.

A preferred embodiment of the invention is a method for the preparation of a vaccine against *S. pneumoniae* comprising the steps of :

a. obtaining a protease maturation protein of *S. pn  moniae* or a fragment thereof or homologous or functionally homologous protein or fragment thereof; and

b. combining the protein or the fragment obtained under (a) with a suitable carrier or adjuvant.

The invention further provides a method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of the vaccine of the invention. The vaccine is suitable for vaccination against all strains and subspecies of *S. pneumoniae,* also for veterinary purposes.

Sub B3 The invention provides for the use of homologous Pmp proteins or fragments thereof of other *S. pneumoniae* species with amino acid sequences or fragments thereof such as peptides that are functionally homologous to the sequence depicted in fig 1B. Said functional homologous peptides can be used in a vaccine for the treatment, preferably the preventive treatment of a wide variety of strains and (sub)species of *S. pneumoniae.*

In one aspect of the invention the antibodies raised against the protein of the present invention may also provide for neutralising effects. These antibodies do not raise any opsonophagocytic activity against *S. pneumoniae* or only to a reduced extent These antibodies merely block certain epitopes of the antigen (in this case Pmp) and may disturb secretion, protection or activation of proteins, directly or indirectly involved in pneumococcal pathogenesis aspects, including colonisation and other processes of *S. pneumoniae.* This provides for an alternative way of treating *S. pneumoniae* infections.

The sequence of the *S. pneumoniae* nucleotides 820800-821738 on contig 3836 (previously known as 7632-8597 on contig 33) and the encoding polypeptide sequence harbouring Pmp are known. The nucleic acid sequence can be used to encode for Pmp or a fragment thereof. By incorp rating this sequenc  r part thereof in a suitable vector and expressing that vector in a cell, it is possible and within the scope of th

invention to obtain recombinant peptide sequences which can subsequently be used in the preparation of a vaccine.

Accordingly the invention also relates to the use of the nucleic acid sequence or fragment thereof or a (functionally) homologous sequence or fragment thereof encoding for Pmp or a fragment thereof. The invention also provides a method for the preparation of a vaccine against *S. pneumoniae*. The method comprises the principal steps of isolating the Pmp protein or the fragment thereof, determining the immunogenic response by raising antibodies against the protein or the fragment, and testing the antibodies in *S. pneumoniae* strains. The invention also provides the recombinant protein or fragment thereof, that has been obtained, for instance, through the expression of a gene sequence encoding for the protein in a suitable vector. The invention also provides for a method of obtaining an antibody and to the antibody. An embodiment of the invention is therefor a method for obtaining an antibody against protease maturation protein comprising the steps of isolating a protease maturation protein or a fragment thereof, raising antibodies against the protein or fragment thereof and isolating the antibodies. The protein or fragment that is used in the preparation of the vaccine or in obtaining the antibody can be a recombinant or synthetic protein or fragment of Pmp.

In an embodiment of the invention, the vaccine can also be derived from the expression of recombinant nucleic acids. The Pmp gene of *S. pneumoniae* can suitably be expressed in *E. coli*.

Pmp and derivatives such as fragments for instance in the form of oligopeptides and modified oligopeptides are tested in animal models to elicit the protection against the different forms of infection (otitis media, pneumonia, sepsis, meningitis) and colonisation.

The production of Pmp for vaccine purposes is in a recombinant form wherein the gene encoding for Pmp is overexpressed in gram positive and/or gram negative bacteria. This yields Pmp in bulk quantities after which further necessary steps such as purification follow.

The present invention further pertains to a method for the identification of proteins expressing opsonophagocytic activity comprising extraction of, preferably surfac associated, proteins, subjecting the obtained proteins to protein

electrophoresis, preferably 2D, obtaining antisera against the proteins, and subjecting the antibodies to an opsonophagocytic assay.

The method provides for a rapid and efficient screening of a large number of proteins or fragments thereof and allows for the rapid identification of proteins of interest. The method according to this aspect of the invention is surprising in that the combination protein electrophoresis and an opsonophagocytic assay results in proteins that are considered to have immunoprotective properties. Electrophoresis techniques use denatured proteins. Antibodies that are active in opsonophagocytic assays are preferably directed to epitopes of the native protein. It is a surprising aspect of the present invention that by the combination of these two methods, antibodies are obtained that allow for immunoprotective properties.

Alternatives for the opsonophagocytic assay are *in vivo* passive immunoprotection assay, *in vivo* active immunoprotective assay and *in vivo* active immunoprotective assay. These techniques are by itself well known in the art and may also serve to identify vaccine candidates according to the invention. By varying the extraction techniques, for instance by varying the detergent or by using chromatographic techniques such as column chromatography, protein fractions of varying composition can be isolated which can be further processed according to the method. It is likewise possible to directly identify the proteins after the electrophoresis step, prior to assaying the proteins for instance by using mass-spectroscopic techniques such as Maldi-tof.

Sub B4

Description of the Figures:

Figure 1 : the *S. pneumoniae* nucleotides 820800-821738 on contig 3836 (http://www.tigr.org/data/S.pneumoniae /) (A) and the encoding polypeptide sequence (B) harbouring Pmp. The presumed methionine start codon of Pmp is depicted in bold and underscored.

Sub B5 Figure 2 : The protease maturation protein of *Lactobacillus paracasei* (Swiss Prot acc. nr. Q02473).

Sub B6 Figure 3 : The protease maturation protein of *Lactococcus lactis subspec. lactis* (Swiss Prot acc. nr. P15294)

Sub B7 Figure 4 : The protease maturation protein of *Lactococcus lactis subsp. cremoris* (Swiss Prot acc. nr. P14308)

## MATERIALS AND METHODS

### **Extraction of surface-associated, hydrophobic proteins of *S. pneumoniae*.**

*S. pneumoniae* FT231 and *S. pneumoniae* EF3296 were cultured at 37 °C in Todd Hewitt broth (Difco laboratories, Detroit, USA) supplemented with 0.5% Yeast Extract (Difco laboratories). At logarithmic growth phase ($OD_{550}$=0.3) the bacteria were harvested by centrifugation, and washed three times with phosphate-buffered saline pH 7.5 (PBS). After the final washing the bacteria were resuspended in TE-buffer (10 mM Tris-Cl, 1 mM EDTA). The cells were disrupted by ultrasonic treatment (Branson sonifier 250, Branson Ultrasonics, Danburry, USA).

Extraction with sulfobetaïne 14 (SB14) was performed as described by Schouls *et al.* (22). In brief, the water-soluble cytoplasmic proteins were removed by washing the bacterial lysates five times with PBS. Cell walls, membranes and other particulate material were collected by centrifugation at 48,400*g for 20 min. Pellets were resuspended in 150 mM NaCl and centrifuged for 20 min at 48,400*g. The pellets were then incubated for 2 hours at room temperature with 0.25% N-tetradecyl-N,N-dimetylammonio-1-propanesulfonate (SB14, Serva, Heidelberg, Germany) in the presence of 150 mM NaCl, 10 mM $MgCl_2$ and 10 mM Tris-HCl pH 8.0 during constant stirring. The hydrophobic, membrane-associated proteins were recovered as described by Wessel and Flügge (23).

Extraction with Triton X114 (Sigma, St. Louis, USA) was also performed as described by Schouls *et al.* (24). Briefly, bacterial lysates were centrifuged at 20,000*g for 20 min. Pellets were dissolved with 1% Triton X114 in PBS for 1 hour at 0 °C.

After xtraction, the suspensions were centrifuged at 25,000* g at 4 °C for 1 hour, the supernatants were incubated at 37 °C for 30 min, and centrifuged at 25,000*g at 25 °C for 1 hour to separate the detergent phase and aqueous phase. The proteins in the detergent phase were extracted according to the procedure of Wessel and Flügge (23). Protein concentrations were measured by the method of Bradford (25).

**Protein electrophoresis and staining.**

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the Biorad minigel system with 13% polyacrylamide gels. The samples were dissolved in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 10 mM DTT, 1% glycerol, 0.01% bromophenol blue indicator (Merck, Darmstadt, Germany), boiled for 5 min and subjected to electrophoresis (26).

Two-dimensional SDS-PAGE was performed according to the instructions of the manufacturer (Pharmacia Biotech, Uppsala, Sweden) including modifications of Rabilloud *et al.* (27). After isoelectric focusing, proteins were separated using gradient (12-20%) polyacrylamide gel electrophoresis.

Silver staining of polyacrylamide gels was performed as described by Blum *et al.* (28). In addition, standard procedures were used to stain the polyacrylamide gels using Coomassie brilliant blue (CBB) (26).

The software program PD Quest (PDI, New York, USA) was used for the computerised analysis of two-dimensional SDS-PAGE gels.

**Hyperimmune rabbit antiserum.**

Hyperimmune antiserum was raised against the hydrophobic, surface-associated proteins by injecting New-Zealand White rabbits subcutaneously into 4-5 places. The SB14 and Triton X114-extracted hydrophobic surface-associated proteins (500 µg) of *S. pneumoniae* FT231 and EF3296, respectively, were dissolved in 0.5 ml 0.9% NaCl, and subsequently mixed with 0.5 ml Freund's incomplete adjuvant (Pierce, Rockford, USA). In addition, hyperimmun rabbit serum was raised against SB14-purified hydrophobic surface-associated pr teins of *S. pneumoniae* FT231 that

were subjected to 1D-SDS-PAGE. The total protein pool was cut from the polyacrylamide gel, washed three times with 0.1 M NaAc, 96% EtOH, ground into a fine suspension in 0.5 ml PBS, and subsequently mixed with 0.5 ml Freund's incomplete adjuvant. Negative control serum was gained by injection of washed and ground polyacrylamide in 0.5 ml PBS mixed with 0.5 ml Freund's incomplete adjuvant. The primary injection was followed by four subcutaneous booster injections at four-week intervals.

Antibodies to type 2 capsule were purchased from Statens Seruminstitut, Copenhagen, Denmark. Recombinant pneumolysin was used to raise hyperimmune sera in rabbits as described previously (29). These sera were used as positive controls in passive immunisation experiments.

**Indirect immuno-cytometric assay.**

Pneumococci were grown to logarithmic phase in Todd-Hewitt broth supplemented with 0.5% Yeast Extract at 37 °C using 5% $CO_2$, then washed three times in ice-cold PBS and stored overnight at 4 °C. The bacteria were incubated in 5% rabbit serum ($10^7$ bacteria in 20 µl final volume) for 15 min at 4 °C while shaking. The bacteria were washed twice using ice-cold PBS and incubated for 15 min at 4 °C with 20 µl (1:5 dilution) of fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, USA) while shaking. The bacteria were washed twice with ice-cold PBS and resuspended in 100 µl of ice-cold paraformaldehyde (0.5 %) in PBS. The samples were analysed in a FACScan flow cytometer (Becton Dickinson, Mountain View, USA).

**Phagocytosis assay.**

Analysis of the opsonophagocytic activity of the sera was performed as described by Alonso Develasco *et al.* (30). In brief, *S. pneumoniae* was grown to logarithmic phase in Todd-Hewitt broth supplemented with 0.5% Yeast Extract at 37 °C using 5% $CO_2$. After washing with PBS, the bacteria were labeled with fluor scein-isothiocyanate (FITC, isomer I, Sigma Chemical Co., St. Louis, USA) (0.5 mg/ml in

PBS) for 1 hour at 4 °C, washed twice and resuspend d in Hank's balanced salt solution (HBSS) containing 1% w/v bovine BSA. The bacteria ($10^8$ bacteria per 100 μl BSA-HBSS) were stored at

-20 °C. Samples of 2.5 * $10^6$ bacteria were transferred into round-bottom microtiter plates (Greiner Labortechnik, Alphen a/d Rijn, The Netherlands). Rabbit sera diluted in BSA-HBSS and heat-inactivated for 30 min at 56 °C were added to the bacteria (final volume 50 μl). The opsonisation was performed at 37 °C for 30 min while shaking. Plates were then placed on ice and 2.5 * $10^5$ human polymorphonuclear cells isolated from peripheral blood of healthy volunteers were added to each well (final volume 100 μl). Human PMNs were isolated by mixing 30 ml of heparinised blood with 30 ml of phosphate-buffered saline (pH 7.4), layered on Ficoll-Paque, and centrifuged for 20 min at 400*g. The lowest layer containing PMNs and erythrocytes was washed once in RPMI (Gibco BRL, Life Technologies LTD, Paisley, UK) containing 0,05% human serum albumin. The erythrocytes were lysed using ice-cold lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4). Phagocytosis was performed for 30 min at 37 °C while shaking. After washing twice with ice-cold HBSS, samples were resuspended in 200 μl of HBSS. The PMNs were fixed by adding 100 μl PBS-2% paraformaldehyde, and the samples were analysed in a FACScan flow cytometer (Becton Dickinson). Fluorescent PMNs observed after opsonisation with antiserum indicates both uptake and binding (referred to as phagocytosis) of FITC-labelled bacteria. The opsonophagocytic activity is defined as the reciprocal of the serum concentration at which 25 % of the human PMNs were fluorescent.

**Immuno electron microscopy.**

Immuno electron microscopy was performed according to the standard operational procedures of the national institute for biological standards and control, Potters bar, United Kingdom.

**Purification, tryptic digest and mass spectrometric analysis of the proteins.**

The protein gel spots of interest were excised from the gel. The gel fragments were sliced thinly and washed twice for 15 minutes in 5 % trichl ro acetic acid

($C_2HCl_3O_2$; Merck, Darmstadt, Germany) and three times in distilled water. The gel fragments were equilibrated in sample buffer pH 6.8 (0.1 % SDS, 10 % glycerol, 50 mM DTT, 12 mM Tris-HCl, 0.01 % bromophenol-blue) for 1 hour at room temperature.

The proteins were concentrated by an agarose electrophoresis (1 % agarose type VIII, Sigma, St. Louis, USA) method as described by Rider et al. (Rider, M. H., M. Puype, J. van Damme, K. Gevaert, S. de Boeck, J. D'Alayer, H. H. Rasmussen, J. E. Celis, and J. Vanderkerckhove. 1995. An agarose-based gel-concentration system for microsequence and mass spectrometric characterization of proteins previously purified in polyacrylamide gels starting at low picomole levels. Eur. J. Biochem. 230:258-265.) and Gevaert et al. (Gevaert, K., J. Verschelde, M. Puype, J. van Damme, M. Goethals, S. de Boeck, and J. Vanderkerckhove. 1996. Structural analysis and identification of gel-purified proteins in the femtomole range, using a novel computer program for peptide sequence assignment, by matrix-assisted laser desorption ionisation-reflection time-of-flight-mass spectrometry. Electrophoresis. 17:918-924) on a Bio-Rad model 150-A gel electrophoresis cell (Bio-Rad laboratories, Richmond, USA) with Pasteur pipettes. After staining the agarose gel with carconcarboxylic acid (Sigma), the proteins were excised from the gel. The agarose fragments were washed with distilled water, and resuspended in 18 μl of digestion buffer pH 8.0 (50 mM $NH_4HCO_3$, 5 mM $CaCl_2$). The agarose was melted at 85 °C for 1 minute. After cooling down to 37 °C 0.05 μg/μl trypsin (trypsin modified sequencing grade, Promega, Madison, USA) was added to digest the proteins for at least 15 hours at 37 °C. Trypsin was inactivated by adding 1 μl of 10 % trifluoro acetic acid ($C_2HF_3O_2$; Merck).

The tryptic digests were analysed using a reversed phase micro-capillary column switching HPLC system (Meiring, H. D., B. M. Barroso, E. van der Heeft, G. J. ten Hove, and A. P. J. M. de Jong. 1999. Sheathless *Nano*flow HPLC-ESI/MS(n) in Proteome Research and MHC Bound Peptide Identification. In Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, Texas.; van der Heeft, E., G. J. ten Hove, C. A. Herberts, H. D. Meiring, C. A. C. M. van Els, and A. P. J. M. de Jong. 1998. A microcapillary column switching system HPLC-electrospray i nisation MS system for th direct identification of peptides presented by maj r histocompatibility complex class I molecules. Anal. Chem. 70:3742-3751.).

Peptide sequencing was perform d on a LCQ quadropole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). Tandem mass spectrometric data were collected in data dependent scan mode for sequence information of single tryptic digest products. With Peptide Search (Mann, M., and M. Wilm. 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal. Chem. 66:4390-4399.), the deduced (partial) amino acid sequences were analysed for matching sequences in all possible translation products of the most current version of the unfinished pneumococcal genome released by The Institute for Genomic Research (TIGR; http://www.tigr.org/data/s_pneumoniae/) to identify the proteins. With the BLAST algorithm (Altschul, S. F., G. W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 251:403-410), putative pneumococcal proteins were analysed for similarity to sequences deposited in the November 1999 version of the non-redundant protein database at the National Center for Biotechnology Information (Washington D.C., USA).

Further proof of principle can be obtained by immunisation experiments in various animal models (mice, rats, rabbits) using purified Pmp, recombinant Pmp or derivatives ad fragments of Pmp.

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